

Spring 5-2016

Elucidation of the Triacylglycerol Biosynthetic Pathway within the Leaves of Transgenic Tobacco Plants

Hunter M. Hawkins
University of Southern Mississippi

Follow this and additional works at: https://aquila.usm.edu/honors_theses

Recommended Citation

Hawkins, Hunter M., "Elucidation of the Triacylglycerol Biosynthetic Pathway within the Leaves of Transgenic Tobacco Plants" (2016). *Honors Theses*. 406.
https://aquila.usm.edu/honors_theses/406

This Honors College Thesis is brought to you for free and open access by the Honors College at The Aquila Digital Community. It has been accepted for inclusion in Honors Theses by an authorized administrator of The Aquila Digital Community. For more information, please contact Joshua.Cromwell@usm.edu.

The University of Southern Mississippi

Elucidation of the Triacylglycerol Biosynthetic Pathway
within the Leaves of Transgenic Tobacco Plants

by

Hunter Hawkins

A Thesis
Submitted to the Honors College of
The University of Southern Mississippi
in Partial Fulfillment
of the Requirements for the degree of
Bachelor of Science
in the Department of Chemistry and Biochemistry

May 2016

Approved by

Philip Bates, Ph.D., Thesis Advisor
Professor of Biochemistry

Sabine Heinhorst, Ph.D., Chair
Department of Chemistry and
Biochemistry

Ellen Weinauer, Ph.D., Dean
Honors College

Abstract

The demand for plant oils is increasing exponentially, which poses a problem as the resources required to produce plant oils are limited. In an effort to alleviate this problem, transgenic tobacco plants that are producing TAG yields of 15% by weight in non-seed tissues have been engineered (Vanhercke *et al.* 2014). In order to achieve even higher oil yields, the pathway that contributes to TAG synthesis in the leaves of these plants needed to be determined. There are three potential pathways that could contribute to TAG synthesis. In order to determine which of these was the major contributor, metabolic tracing was used to measure the flux of intermediates through TAG metabolism.

[¹⁴C]acetate was used to trace lipid metabolism in developing “wild type” and “high oil” plants. This radiolabeled acetate incorporated into TAG metabolism via fatty acid synthesis, labeling newly synthesized fatty acids with [¹⁴C]. The results of this experiment revealed that in wild type tissue, fatty acids fluxed through intermediates and primarily ended up in MGDGs, but in high oil tissue, fatty acids fluxed through PCs before incorporating into TAGs. Incorporation of radiolabel into PCs at early time points suggested that fatty acids participate in acyl editing before being esterified to precursors in the synthesis of TAGs. This revealed that leaf oil and membrane lipid synthesis are overlapping biosynthetic pathways. In future experiments, [¹⁴C]glycerol tracing will be conducted to further elucidate the pathway contributing to TAG synthesis in the leaves of transgenic tobacco plants.

Keywords: plant oil, lipid, fatty acid, metabolism, radiolabel

Table of Contents

List of Figures	vi
Chapter 1: Introduction	1
1.1: Plant Oil Background.....	1
1.2: TAG Metabolism	2
1.3: Engineering of Transgenic Tobacco Plants	4
1.4: Determining the Pathway of TAG Biosynthesis.....	6
Chapter 2: Experimental	8
2.1: Radiolabeled Metabolic Tracing Experiments	8
2.2: Lipid Extraction	8
2.3: TLC and Phosphor Imaging Analyses	9
Chapter 3: Results	12
3.1: Lipid Quantification.....	12
3.2: Radioactivity Incorporated into Each Plant Type.....	13
3.3: Flux of Fatty Acids Through Lipid Intermediates	13
Chapter 4: Conclusions	16
References.....	19

List of Figures

Figure 1: TAG Synthesis Flux Model.....	2
Figure 2: Genes Involved in TAG Accumulation in Transgenic Tobacco Plants	5
Figure 3: Potential Pathways of TAG Biosynthesis	7
Figure 4: Neutral Lipid TLC Plates	10
Figure 5: Polar Lipid TLC Plates.....	11
Figure 6: FAME in Each Sample.....	12
Figure 7: Incorporation of Radioactivity over Time	13
Figure 8: Flux of Metabolites Through Wild Type Tissue	14
Figure 9: Flux of Metabolites Through High Oil Tissue	15

1. Introduction

1.1 Plant Oil Background

Plant oils are present in many everyday products. They are utilized in everything from cooking sprays to fuel production. With each technological stride, the number of uses for plant oils increases. Over the past twenty years, the demand for plant oils has increased significantly, and the current demand is projected to increase at an exponential rate over the next several decades (Carlsson *et al.* 2011). Due to this rise in demand, the production of these plant oils has become an increasingly popular field of study. However, land area, fertilizer, and other resources required to produce plant oils are limited. This will likely present a problem in the near future. It is possible that the production of plant oils will not be able to keep up with the demand for those oils. Currently, there are several solutions that are being sought to overcome this potential problem. One of these potential solutions is to increase the efficiency of oil production in plants per acre of crop land.

Plant oils are made of triacylglycerols (TAGs). In wild plants, nearly all of the TAGs are located in the seeds of the plant; however, in recent experiments, high oil yields have been produced in the non-seed tissue of plants, such as leaves. This was accomplished through the overexpression of genes that control lipid synthesis and accumulation in leaves (Vanhercke *et al.* 2014). This is significant in many ways. Seeds make up a small portion of the plant, so increased oil production in non-seed tissue could lead to much higher oil yields per acre of crop land. This recent breakthrough in production efficiency could be a significant step in compensating for the lack of resources required to supply oils; however, further improvements in production

efficiency still need to be made. In order to accomplish these improvements, oil synthesis in plant leaves needs to be better understood.

1.2 TAG Metabolism

TAG is a lipid that can be found in just about any eukaryotic organism, but the unsaturated forms of TAG found in plants are the forms that are most valuable (Bates and Browse 2012). TAG is composed of a glycerol backbone that has three fatty acid chains esterified to it. TAG synthesis is complicated and can be achieved through several different pathways as shown in Figure 1.

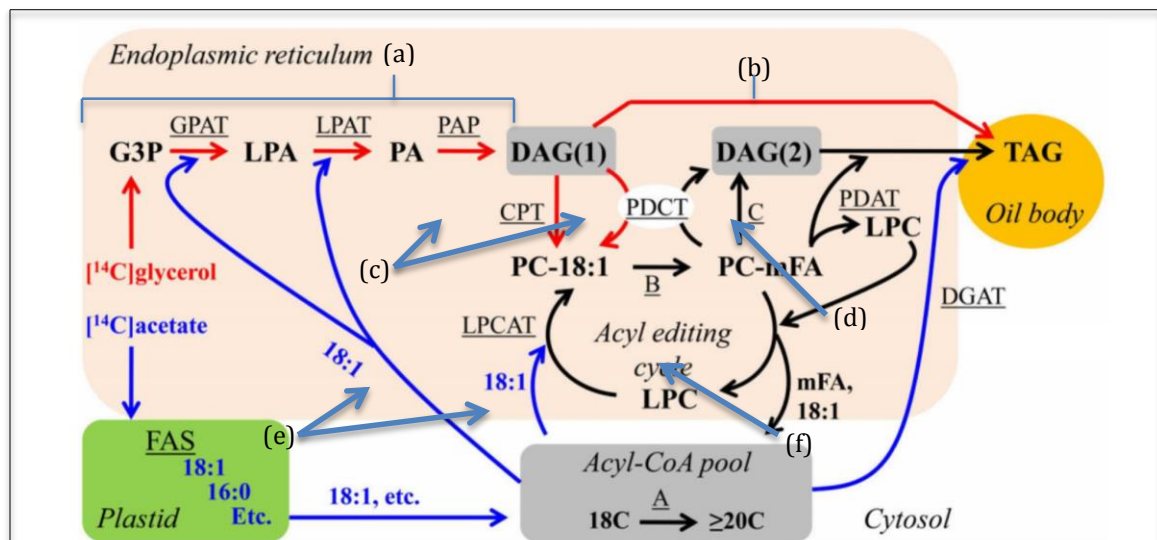


Figure 1: TAG Synthesis Flux Model

This figure shows the pathways that contribute to oil biosynthesis.

- (a) Fatty acids are esterified to G3P to form *de novo* DAG.
- (b) *De novo* DAG is converted to TAG by the addition of a third fatty acid.
- (c) *De novo* DAG is converted to PC by the addition of a choline group
- (d) PC is converted to PC-derived DAG by the removal of a choline group
- (e) Fatty acids from the acyl-CoA pool enter TAG metabolism
- (f) Acyl editing modifies fatty acids attached to PC

Substrate abbreviations: G3P, glycerol-3-phosphate; LPA, lyso-phosphatidic acid; PA, phosphatidic acid; DAG, diacylglycerol; PC, phosphatidylcholine; mFA, PC-modified FA; TAG, triacylglycerol. Underlined enzymatic reactions: CPT, CDP-choline:DAG cholinephosphotransferase; DGAT, acyl-CoA:DAG acyltransferase; FAS, fatty acid synthesis; GPAT, acyl-CoA:G3P acyltransferase; LPAT, acyl-CoA:LPA acyltransferase; LPCAT, acyl-CoA:LPC acyltransferase; PAP, PA phosphatase; PDAT, phospholipid:DAG acyltransferase; PDCT, PC:DAG cholinephosphotransferase.

Modified from "The Significance of Different Diacylglycerol Synthesis Pathways on Plant Oil Composition and Bioengineering" (p. 6), by P. D. Bates and John Browse, 2012, *Frontiers in Plant Science*, 3, p. 6.

In TAG synthesis, the immediate precursor to TAG is DAG. Therefore, in order to make TAGs, DAGs must first be synthesized. As seen in Figure 1, there are two DAG pools that contribute to TAG synthesis: the *de novo* DAG pool, DAG(1), and the PC-derived DAG pool, DAG (2). The former involves the synthesis of DAGs from glycerol-3-phosphate (G3P) and acyl-CoAs (Figure 1a), and the latter involves the synthesis of DAGs from PCs through the removal of its phosphocholine (Figure 1d). In some plants, *de novo* DAG is the only intermediate in TAG synthesis, while in others, PC-derived DAG is the predominant intermediate, producing over 90% of TAGs found in the plant (Bates *et al.* 2013).

In order to produce DAGs through *de novo* synthesis, fatty acids must first be synthesized. This process is performed within the plastids of the plant cell. The substrate in fatty acid synthesis is acetyl-CoA. In *de novo* synthesis, fatty acids are synthesized two carbons at a time from acetyl-CoA to produce chains of either sixteen or eighteen carbons. The length and degree of unsaturation of the fatty acid chain is determined by the enzymes that act on the chain. Once these chains have been synthesized, they are taken from the plastid to the endoplasmic reticulum (ER). This is where TAG synthesis occurs (Bates *et al.* 2013).

Fatty acids are transported from the plastid to the ER in the form of acyl-CoA (Figure 1e). This acyl-CoA is the substrate in the synthesis of lyso-phosphatidic acid (LPA). Acyl-CoA:glycerol-3-phosphate acyltransferase breaks the thioester bond in acyl-CoA and esterifies the fatty acid to glycerol 3-phosphate (G3P), producing LPA. Another fatty acid chain is then esterified to LPA by acyl-CoA:lyso-phosphatidic acid acyltransferase, producing phosphatidic acid (PA). Phosphatidic acid phosphatase then

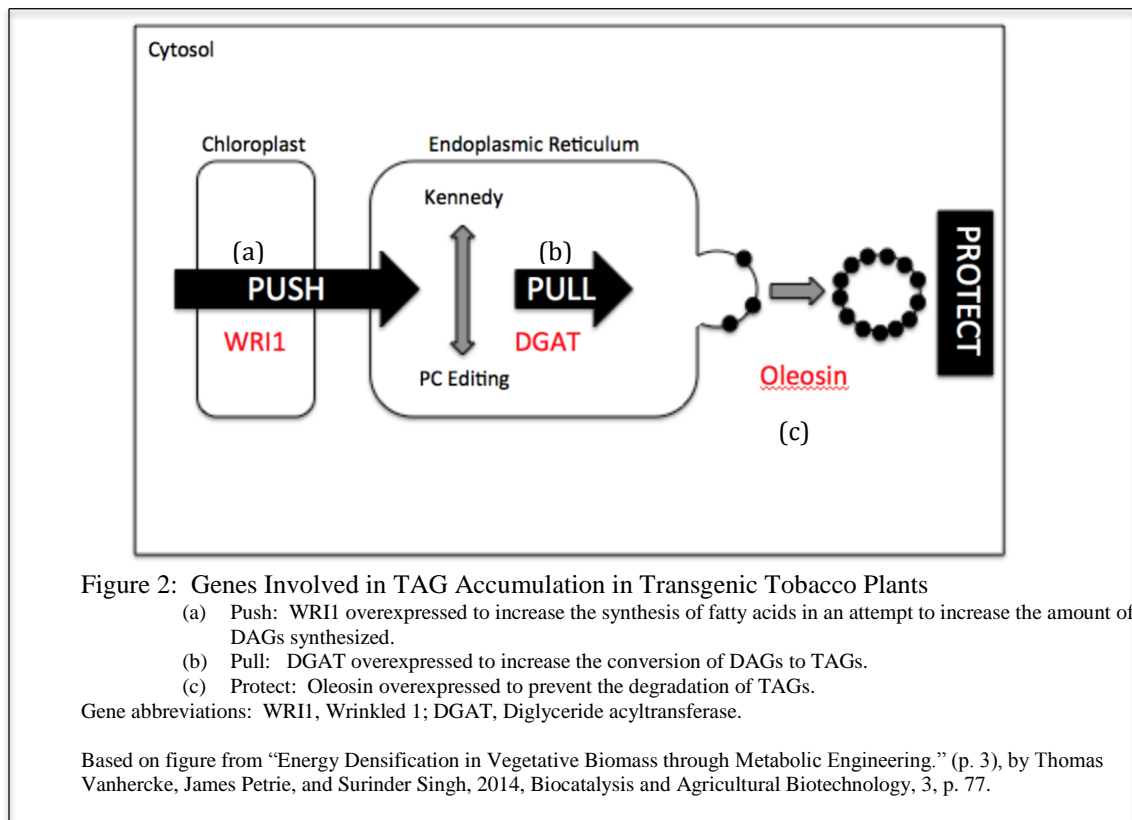
removes the phosphate, producing *de novo* DAGs. These three steps are illustrated in Figure 1a. *De novo* DAGs can then be converted to TAGs by the addition of another fatty acid chain from acyl-CoA by acyl-CoA:diacylglycerol acyltransferase (Figure 1b) (Bates and Browse 2012).

In order for PC-derived DAG synthesis to take place, PCs must first be produced. PC is a membrane lipid that is produced from *de novo* DAGs. There are two pathways in which PC synthesis can occur. The first pathway is catalyzed by CDP-choline:diacylglycerol cholinephosphotransferase (CPT), and the second is catalyzed by phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT) (Figure 1c). Fatty acid desaturation occurs when fatty acids are attached to PCs, so the accumulation of polyunsaturated fatty acids in oil indicates the flux of fatty acids through PCs. PCs also undergo a process called “acyl editing.” Acyl editing is a cycle that removes an acyl residue from PC and replaces it with a new acyl group from the acyl-CoA pool (Figure 1f). There is no net gain or loss of PCs in this cycle. PC can be converted back to DAG through the removal of its phosphocholine group. Due to acyl-editing and fatty acid desaturation, these PC-derived DAGs often contains fatty acid chains that are different than those found in *de novo* DAGs (Bates and Browse 2012).

1.3 Engineering of Transgenic Tobacco Plants

As shown in Figure 2, TAG production can be generalized into three separate phases: push, pull, and protect. The push phase is defined as the *de novo* synthesis of DAGs, occurring in the plastid and ER (Figure 2a). The conversion of DAGs to TAGs is considered to be the pull phase (Figure 2b). The final phase is referred to as the protect phase, and that involves the prevention of TAG degradation (Figure 2c). Typically,

TAGs cannot be extracted from non-seed tissues of a plant; however, through gene modification and metabolic engineering in each of these phases, TAG yields of over 15% by weight have been produced in non-seed tissues such as leaves (Vanhercke *et al.* 2014).



The gene WRI1 encodes a transcription factor that regulates fatty acid biosynthesis. WRI1 was overexpressed in order to increase the amount of fatty acids being synthesized (Figure 2a). This increase in fatty acids most likely led to an increase in *de novo* DAGs produced in non-seed tissues. Since PCs are synthesized from *de novo* DAGs, it is possible that PC-derived DAG synthesis was increased through this gene expression. DGAT1 was also overexpressed in an attempt to increase TAG production in the pull phase of TAG synthesis (Figure 2b). DGAT1 is a gene product that is scarcely produced in leaves but is required for TAG synthesis. The final genes manipulated were oleosin genes (Figure 2c). Oleosin is a protein that coats oil bodies in seeds and is not

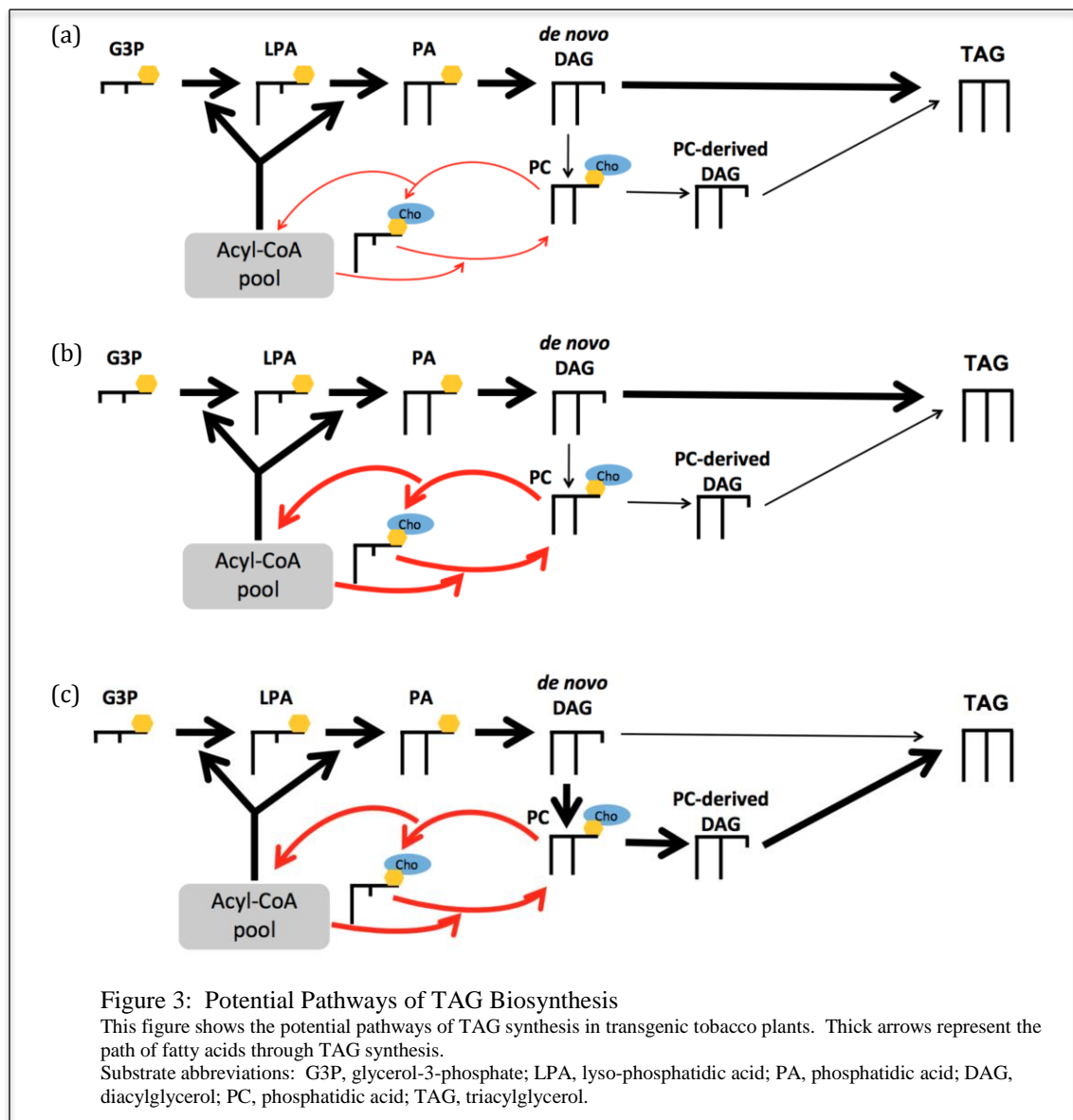
typically found in leaves or other non-seed tissues. This limited the amount of TAGs that is degraded in these tissues and enhanced the accumulation of TAGs. Through the overexpression of these genes, the transgenic tobacco plants produced an average of 15.8% oil by weight in their leaves (Vanhercke *et al.* 2014).

1.4 Determining the Pathway of TAG Biosynthesis

More about the TAG metabolism for both the wild type and transgenic plants needs to be discovered. Hopefully a greater knowledge of metabolism will lead to more efficient metabolic engineering and even higher oil yields. In order to learn more about TAG metabolism, the flux of metabolic intermediates in TAG synthesis must be measured. Radioactive metabolic tracing is a technique that can be used to measure this relative flux of metabolites through competing metabolic pathways (Allen *et al.* 2015). By incubating tissue in growth media containing [^{14}C]acetate, new fatty acids synthesized within the tissue are subsequently labeled. These labeled fatty acids are then incorporated into TAG intermediates and eventually TAGs. By measuring the amount of radioactivity in individual intermediates over time, the pathway through which TAG synthesis is proceeding can be determined.

There are three known potential pathways through which the newly synthesized and [^{14}C] labeled fatty acids can be incorporated into the TAG structure. These known pathways are illustrated in Figure 3. In the first pathway, fatty acids are esterified to G3P, LPA, and DAG in the formation of TAGs through the *de novo* pathway (Figure 3a). In the second pathway, fatty acids are added to PC and are desaturated during acyl editing. After acyl editing, they cycle back into the acyl-CoA pool before being added G3P, LPA, and DAG in the process of forming TAGs (Figure 3b). In the third and final

pathway, fatty acids again participate in acyl editing before cycling back into the acyl-CoA pool. They are then added to G3P and LPA forming DAGs. A choline group is then added to DAG forming PC, where the fatty acids participate in acyl editing again before the choline group is removed, creating PC-derived DAG which can then be converted into TAG (Figure 3c). The pathway contributing to TAG biosynthesis will be determined by measuring the flux of newly synthesized fatty acids through TAG synthesis with [^{14}C]acetate labeling.



2. Experimental

2.1 Radiolabeled Metabolic Tracing Experiments

Three “wild type” tobacco plants and three of the transgenic “high oil” tobacco plants (provided by Vanhercke *et al.* 2014) were grown in a growth chamber. This chamber exposed the plants to 16 hours of white light each day. The chamber was held at 26°C during white light exposure and 22°C during the 8-hour dark period. After 30 days, the labeling experiment was performed. Tissue was removed from each plant in the form of 12 leaf discs measuring 1 cm in diameter. These leaf discs were radiolabeled with [¹⁴C]acetate using the protocol outlined in Bates *et al.* 2007. Three replicates (A, B, and C) for both the wild type and the high oil plants were labeled for time periods of 3, 6, 10, 30, and 120 minutes, respectively. This yielded fifteen wild type samples and fifteen high oil samples. The radioactive [¹⁴C]acetate is incorporated during FA synthesis forming radiolabeled fatty acids. These enter TAG metabolism when fatty acid chains are esterified to G3P and other intermediates (Day and Wilkinson 1967), or through acyl editing (Bates and Browse 2012). At each time point, metabolism was stopped by quenching the tissue in isopropanol at 80°C.

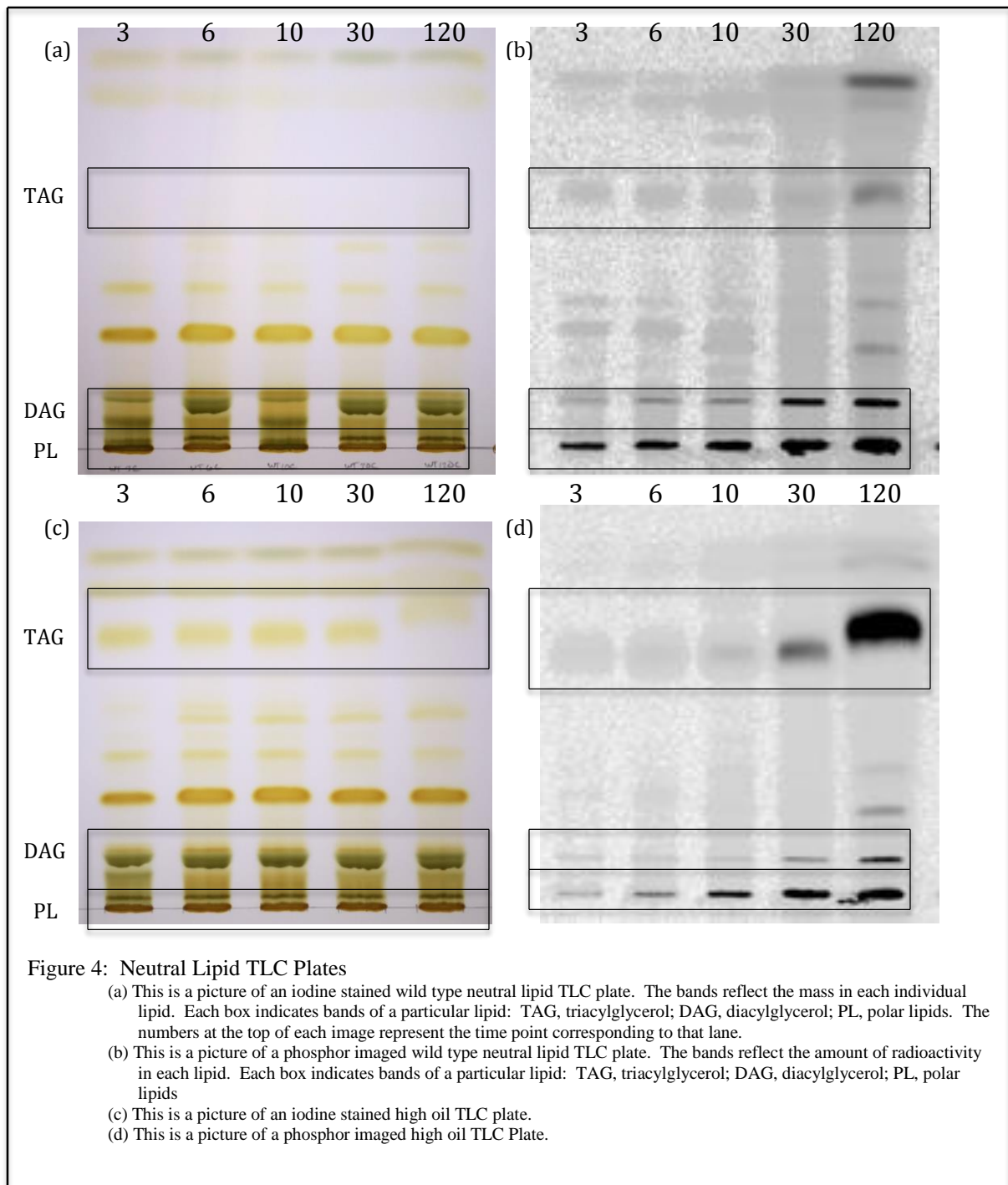
2.2 Lipid Extraction

After labeling, the lipids needed to be extracted from the tissue. The extraction methods outlined in Bates *et al.* 2007 were used to extract the lipids. In brief, the quenched tissue was ground using a mortar and pestle. Then, a mixture of hexane and isopropanol (6:4) was added to separate the samples into an organic phase and an aqueous phase. The organic phase contained the lipids and the aqueous phase contained the aqueous soluble components as well as the remaining unincorporated [¹⁴C]acetate.

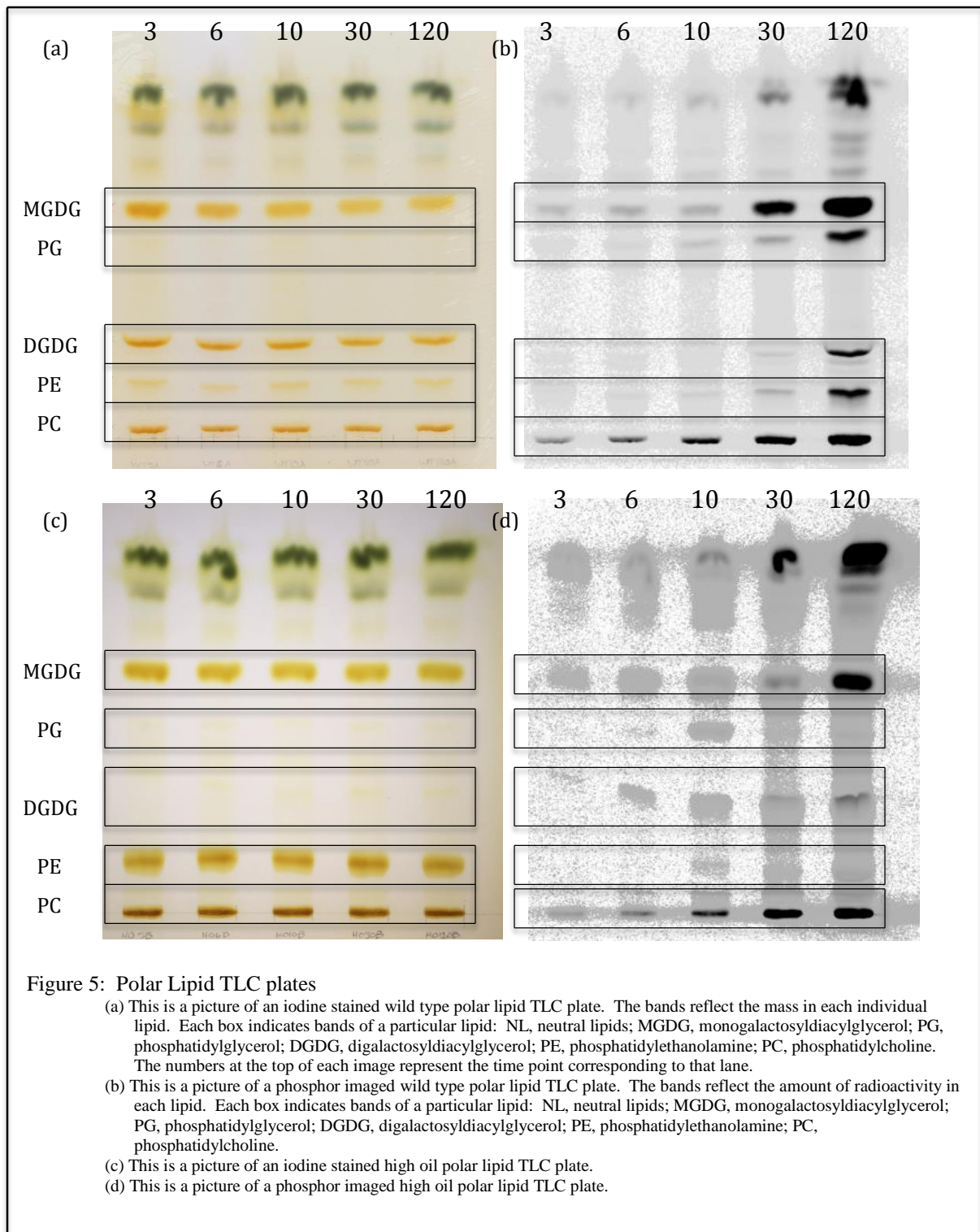
The aqueous phase was discarded, and the organic phase was dried down using nitrogen in order to concentrate the extract. The dried extract was then resuspended in 500 μ L of toluene. A 50 μ L aliquot of each sample was taken and the lipids in those aliquots were transesterified into fatty acid methyl esters (FAMES). These FAMES were then quantified using gas chromatography in order to determine the amount of each type of fatty acid in each individual sample. Another 20 μ L aliquot of each sample was taken and scintillation counting was performed, which quantified the amount of radioactivity in each sample. A final 10 μ L aliquot of each sample was taken and atomic absorption spectroscopy was used to measure the chlorophyll content.

2.3 TLC and Phosphor Imaging Analyses

To understand the differences in fatty acid flux through the lipid metabolic network between the wild type and transgenic leaves, we separated individual lipids by thin layer chromatography (TLC). TLC is a technique that separates compounds based on their polarity using a plate covered with a thin adsorbent material (stationary phase) and an organic solvent (mobile phase). First, neutral lipid TLC was performed to separate the neutral lipids in each sample. This was done using a solvent system of hexane, ether, and acetic acid (70/30/1). This solvent mixture allowed the neutral lipids to separate across the plate with the polar lipids remaining at the bottom of the plate as seen in Figure 4. The iodine stained TLC plates have bands that are equal at each time point, but the phosphor imaged TLC plates have bands representing radioactivity that increase with each time point. These bands represent the newly synthesized lipids.



After these experiments were performed, polar lipid TLC was performed to separate the polar lipids in each sample using a solvent system of acetone, toluene, and water (91/30/7). This solvent mixture allowed the neutral lipids to move to the top of the plate, while the polar lipids separated across the plate as shown in Figure 5.



The protocols used for neutral and polar lipid TLC are outlined in Bates *et al.*

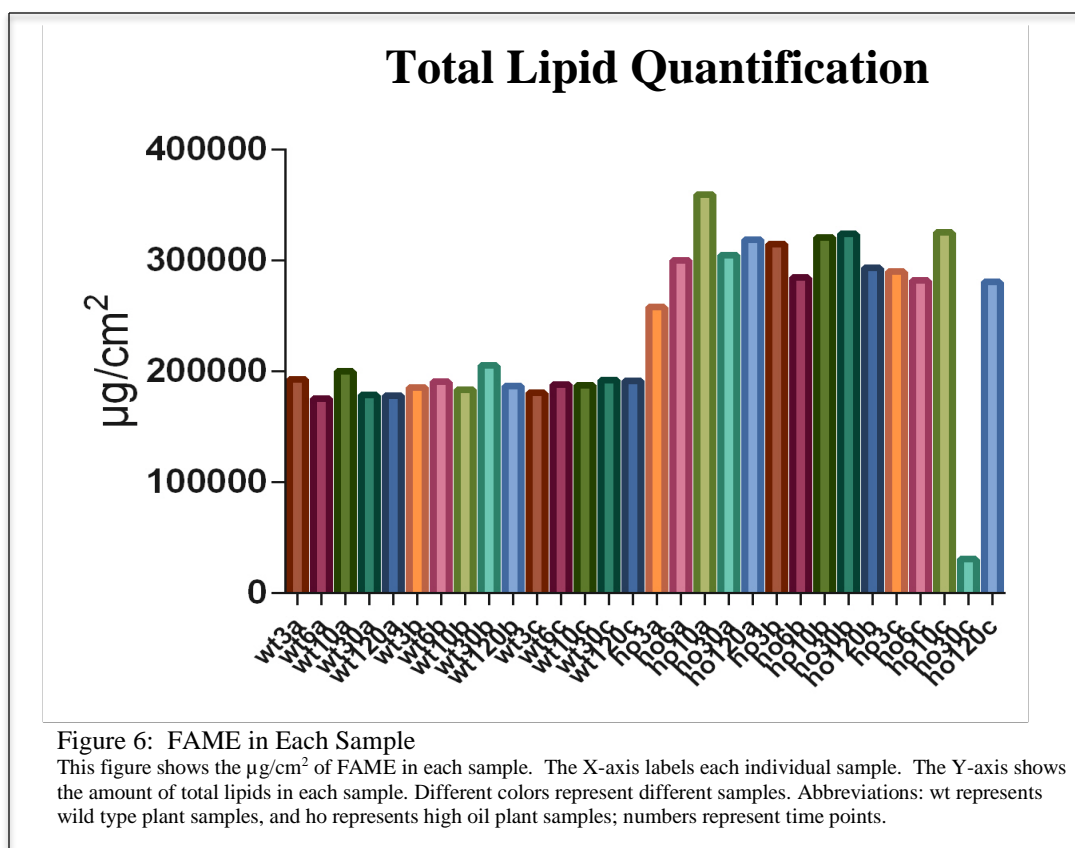
2007. Once the TLC experiments had been performed, the amount of radioactivity of

each specific lipid was quantified using phosphor imaging and the results of the scintillation counting experiment.

3. Results

3.1 Lipid Quantification

The results of the FAME quantification experiment revealed that the high oil tissue had more total lipids than the wild type tissue. This supported the results from the Vanhercke *et al.* 2014 experiments. The amount of fatty acids produced in each sample can be seen in Figure 6.



As one can see, all of the high oil lines, with the exception of high oil 30 c, show a greater amount of FAME than any of the wild type lines. The reason the high oil 30 c line contained significantly less FAME [$\mu\text{g}/\text{cm}^2$] is that during incubation, the cap was not tightened all the way and the sample evaporated off.

3.2 Radioactivity Incorporated into Each Plant Type

Through scintillation counting the disintegrations per minute (DPM), which is a unit of radioactivity, in each sample was measured. Next, spectroscopy was used to measure the chlorophyll content, and the DPM per chlorophyll content was then plotted against time for each sample.

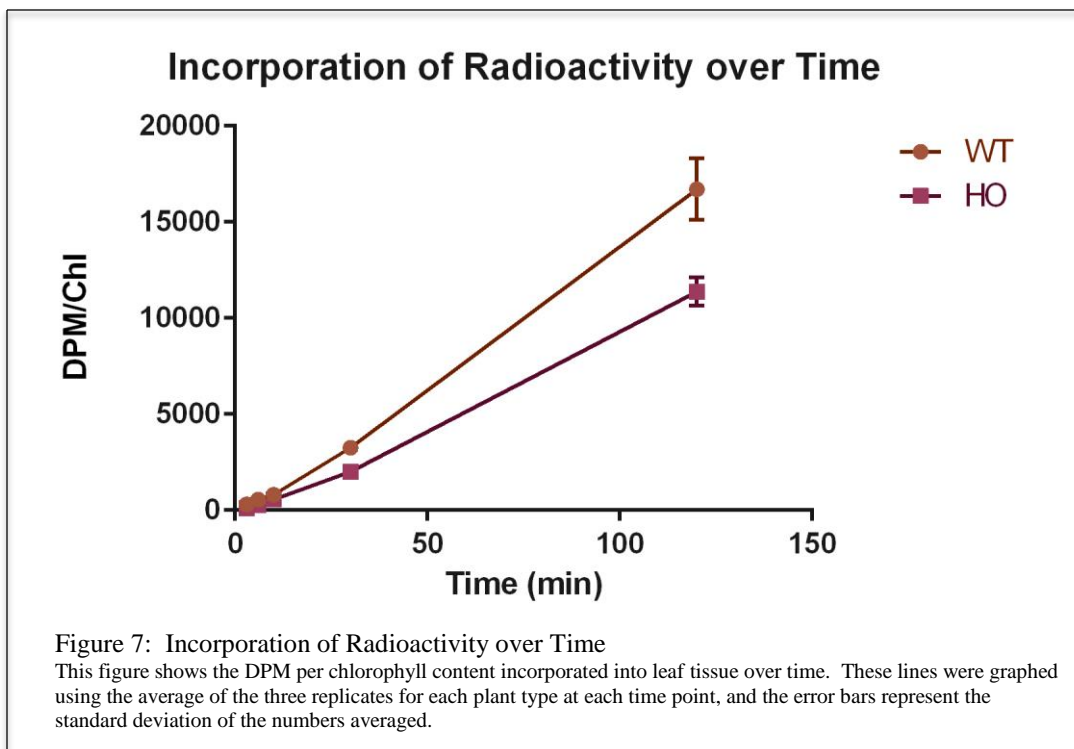


Figure 7 shows that more radioactivity is incorporated into the wild type plants than into the high oil plants. Considering that the high oil plants produce more lipids than the wild type plants, this is a surprising result. This could be due to possible futile cycling of fatty acid synthesis and degradation in the high oil plants.

3.3 Flux of Fatty Acids Through Lipid Intermediates

After the TLC experiments were performed, separating both the neutral and polar lipids in each sample, phosphor imaging as well as the data from the scintillation counting experiment were used to quantify the amount of radioactivity incorporated into

each lipid at each specific time point. This revealed the flux of metabolites during TAG synthesis. These results are represented in Figures 8 and 9.

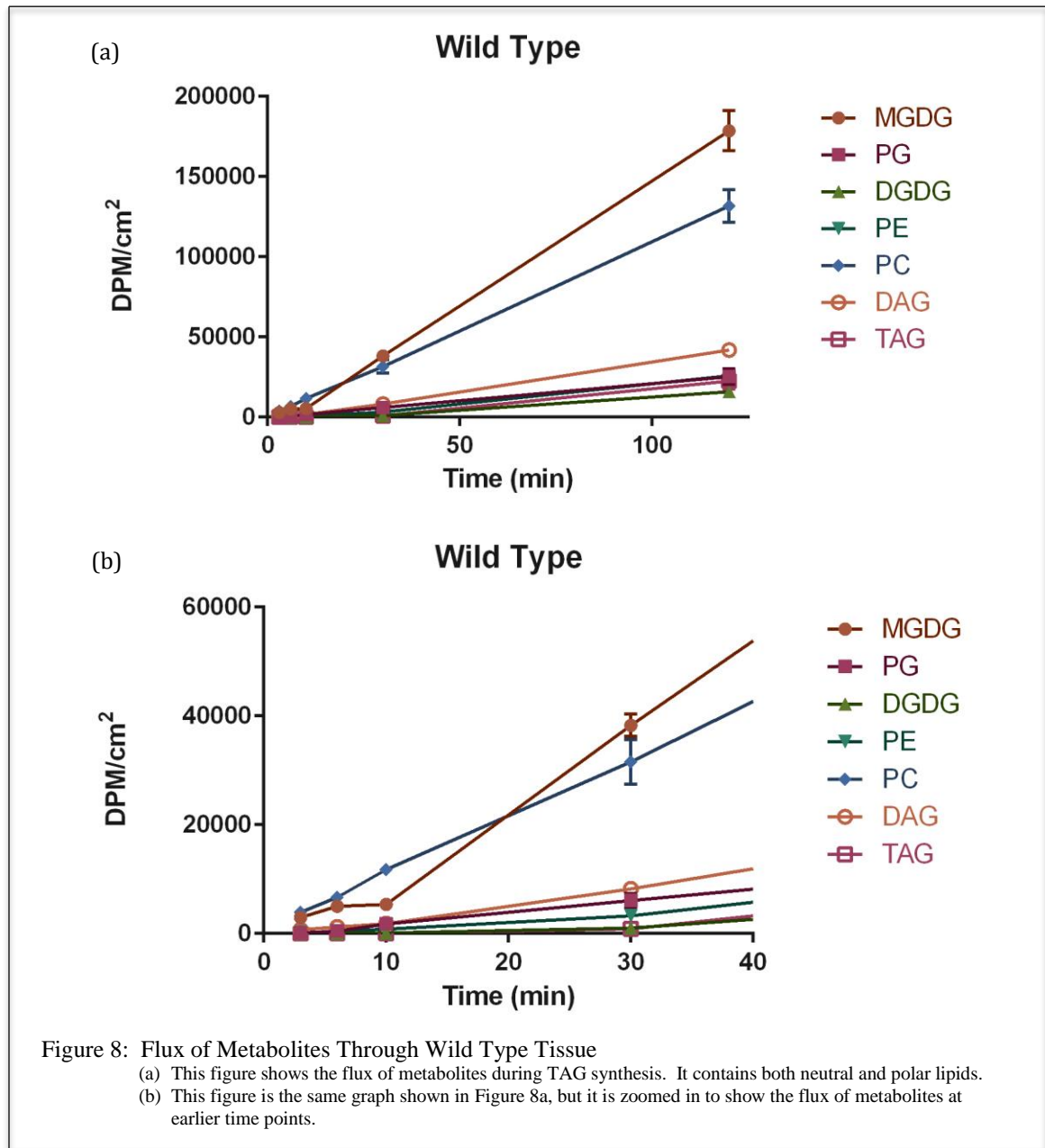
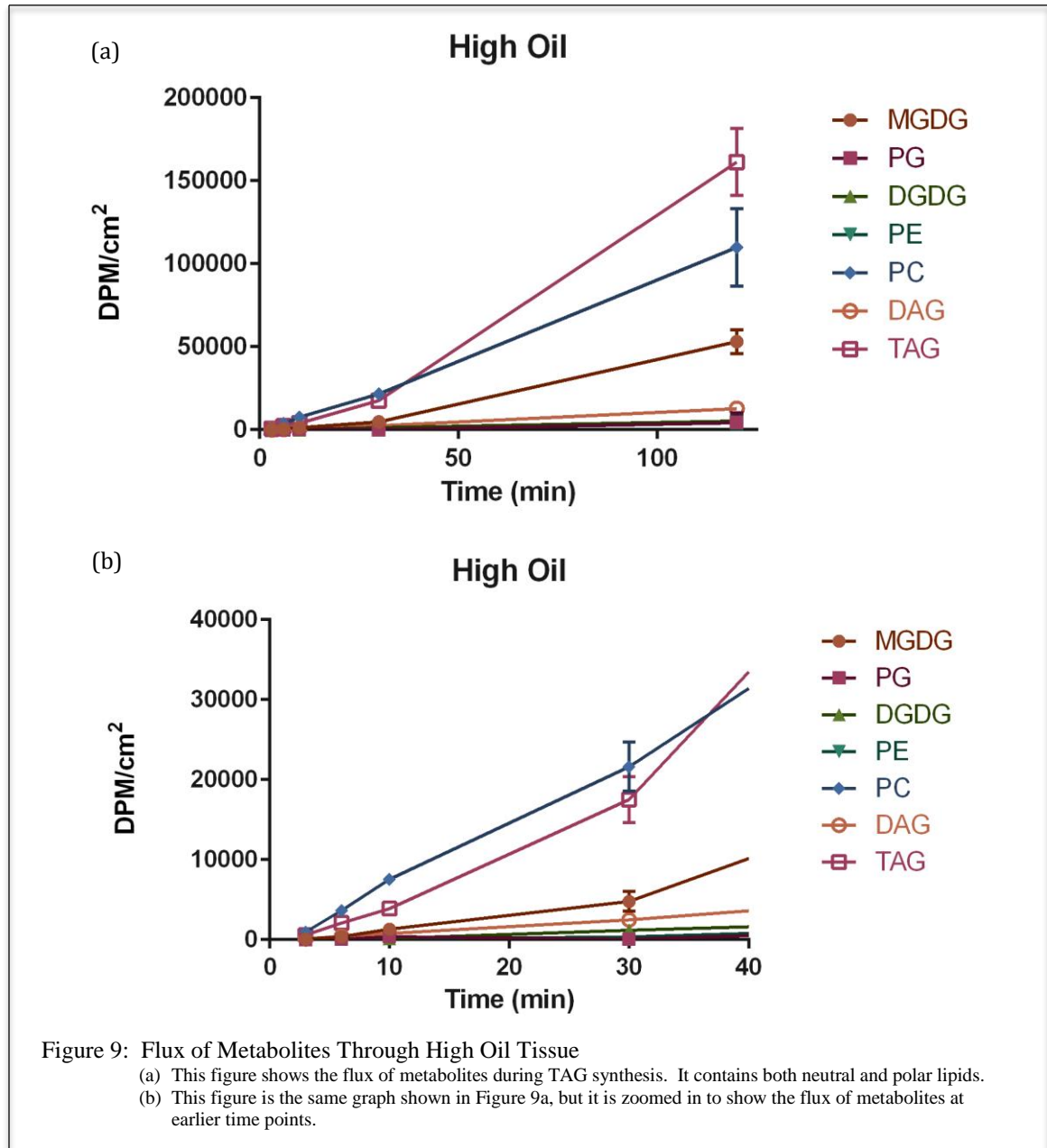


Figure 8 shows that the majority of the radiolabel in wild type plants is incorporated into PCs first, and then as time goes on, more of the radiolabel is incorporated into monogalactosyldiacylglycerol (MGDG). MGDG is a galactolipid that is synthesized in the leaves of plants, so it is not surprising that much of the radiolabel

ends up in MGDGs. Figure 8 also shows that very little of the radiolabel is incorporated into TAGs. This differs from the flux of metabolites in high oil plants, which can be seen in Figure 9.



As seen in Figure 9, the radiolabel in the high oil plants is incorporated into PCs first, and then as time goes on, more is incorporated into TAGs. There is a significant

increase in the amount of radiolabel incorporated into TAGs in the high oil plants. Also, there is a significant decrease in the amount of radiolabel incorporated into MGDGs.

4. Conclusions

As shown in Figure 3, the FAME quantification experiment revealed that the transgenic high oil plants are indeed producing more fatty acids than the wild type plants. However, after performing scintillation counting and measuring the chlorophyll content of each sample, the data shown in Figure 7 suggested that the wild type plants were incorporating more radiolabeled acetate than the high oil plants. Since the high oil plants are producing more fatty acids, one would assume that more radiolabeled acetate would be incorporated into the high oil than into the wild type tissue, but this is not the case. It is likely that the overexpression of certain genes in the high oil tissue has led to futile cycling, where fatty acids are being produced and degraded rapidly, causing less net radiolabel to be incorporated into fatty acids.

The results of the TLC experiments revealed that there is a difference in metabolism between the wild type and the high oil plants. Figure 8 suggests that most of the newly synthesized fatty acids are incorporated into PCs then over time more are incorporated into MGDGs. Very little fatty acids are incorporated into TAGs. This differs from the metabolism in the high oil plants. Like the wild type plants, it appears that fatty acids are incorporated quickly into PCs; however, over time the fatty acids are incorporated into TAGs instead of MGDGs. Due to the high amount of fatty acids incorporated into PCs in early time points, it appears that TAGs are not synthesized via the *de novo* pathway, ruling out the first potential pathway. This means that either the second or third potential pathway is the pathway contributing to TAG synthesis in these

transgenic plants. Fatty acids participate in acyl editing before being incorporated into the TAG structure, but the pathway TAG synthesis takes after acyl editing occurs is still unknown. In order to determine which of the two remaining pathways is the major contributor to TAG synthesis, more experiments must be performed.

In the future, a glycerol labeling experiment will be performed. Radioactive [^{14}C]glycerol will be added to the plant tissue in a procedure similar to the acetate labeling outlined in the Experimental section. This radiolabeled glycerol will be incorporated into TAG synthesis as G3P during synthesis. The incorporation of radioactivity into different intermediates will again be measured. If radioactivity is incorporated into DAGs at a high rate followed by incorporation into TAGs with little incorporation into PCs, this would suggest that the glycerol is used to synthesize *de novo* DAGs, which are then quickly converted to TAGs. This would mean that the second potential pathway is the major pathway of TAG synthesis. On the other hand, if radioactivity is incorporated into DAGs at early time points and PCs at later time points with incorporation into TAGs lagging behind both, it would suggest that the third potential pathway is the major pathway. In this case the glycerol would be used to synthesize DAGs, which would then be converted into PCs before being converted back into PC-derived DAGs and eventually TAGs.

Plant oils are valuable because of their numerous uses. A lack of resources to supply these plant oils could lead to major problems down the road. Fortunately, steps to increase oil production per acre of cropland have been taken, but much more needs to be done. The metabolic pathway responsible for the high TAG yields in transgenic tobacco plants needs to be determined. Once the contributing pathway has been discovered, the

genes required in that pathway can be isolated. Overexpression of these specific genes will hopefully lead to even greater yields of plant oils in the future.

References

- Allen, D.K., P.D. Bates, and H. Tjellström. "Tracking the metabolic pulse of plant lipid production with isotopic labeling and flux analyses: Past, present and future." *Progress in Lipid Research* 58 (2015): 97-120. Print.
- Bates, Philip D., and John Browse. "The Significance of Different Diacylglycerol Synthesis Pathways on Plant Oil Composition and Bioengineering." *Frontiers in Plant Science* 3 (2012): 1-11. Print.
- Bates, Philip D., John B. Ohlrogge, and Mike Pollard. "Incorporation of Newly Synthesized Fatty Acids into Cytosolic Glycerolipids in Pea Leaves Occurs via Acyl Editing." *Journal of Biological Chemistry* 282.43 (2007). 31206-16. Print.
- Bates, Philip D., Sten Stymne, and John Ohlrogge. "Biochemical Pathways in Seed Oil Synthesis." *Current Opinion in Plant Biology* 16 (2013): 358-64. Print.
- Carlsson, Anders S., Jenny Lindberg Yilmaz, Allan G. Green, Sten Stymne, and Per Hofvander. "Replacing Fossil Oil with Fresh Oil - with What and for What?" *European Journal of Lipid Science and Technology Eur. J. Lipid Sci. Technol.* 113.7 (2011): 812-31. Web.
- Day, A. J., and G. K. Wilkinson. "Incorporation of ¹⁴C-Labeled Acetate into Lipid by Isolated Foam Cells and by Atherosclerotic Arterial Intima." *Circulation Research* 21.5 (1967): 593-600. Web.
- Vanhercke, Thomas, James Petrie, and Surinder Singh. "Energy Densification in Vegetative Biomass through Metabolic Engineering." *Biocatalysis and Agricultural Biotechnology* 3 (2014): 75-80. Print.